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Comment:

Past studies have linked human exposure to electromagnetic field (EMF) to leukemia, genotoxicity, brain tumors, and other diseases. One way in which EMF may contribute to these diseases is by effecting the levels of reactive oxygen species (ROS) in some tissues. If too much ROS is being produced in comparison to how efficiently a system can neutralize them, the condition is called oxidative stress, and ROS can damage DNA and oxidize amino acids in proteins. Oxidative stress increase risk of cancer and other diseases. Past research has shown no link between EMF exposure and increased production of ROS. This study aimed to investigate whether EMF can harm the defense mechanisms against oxidative stress, namely the antioxidative capacity of body fluids.

Wistar rats, some of which were injected with Complete Freund's Adjuvant to incite inflammation, were used as the subjects in this experiment. The rats were placed 1 meter from the EMF source, which had a frequency of 1800 MHz. Some rats were given a TRAM injection prior to EMF exposure, which contains the opioid tramadol that is believed to aid defense mechanisms against oxidative stress. After 15 minutes of EMF exposure, blood samples were taken and analyzed for antioxidant capacity.

The study showed that EMF exposure reduced antioxidant capacity in both the healthy rats, and the rats with inflammation. Healthy and inflamed rats who were given the TRAM injection showed slightly higher antioxidant capacity than those who were not given the injection. These results show that EMFs may cause oxidative stress, which could, with further research, provide evidence for EMFs contributing to neurodegenerative diseases.

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Changes in antioxidant capacity of blood due to mutual action of electromagnetic field (1800 MHz) and opioid drug (tramadol) in animal model of persistent inflammatory state

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Abstract:

Background: The biological effects and health implications of electromagnetic field (EMF) associated with cellular mobile telephones and related wireless systems and devices have become a focus of international scientific interest and world-wide public concern. It has also been proved that EMF influences the production of reactive oxygen species (ROS) in different tissues.

Methods: Experiments were performed in healthy rats and in rats with persistent inflammatory state induced by Complete Freund's Adjuvant (CFA) injection, which was given 24 h before EMF exposure and drug application. Rats were injected with CFA or the same volume of paraffin oil into the plantar surface of the left hind paw. Animals were exposed to the far-field range of an antenna at 1800 MHz with the additional modulation which was identical to that generated by mobile phone GSM 1800. Rats were given 15 min exposure, or were sham-exposed with no voltage applied to the field generator in control groups. Immediately before EMF exposure, rats were injected intraperitoneally with tramadol in the 20 mg/kg dose or vehicle in the 1 ml/kg volume.

Results: Our study revealed that single EMF exposure in 1800 MHz frequency significantly reduced antioxidant capacity both in healthy animals and those with paw inflammation. A certain synergic mode of action between applied electromagnetic fields and administered tramadol in rats treated with CFA was observed.

Conclusions: The aim of the study was to examine the possible, parallel/combined effects of electromagnetic radiation, artificially induced inflammation and a centrally-acting synthetic opioid analgesic drug, tramadol, (used in the treatment of severe pain) on the antioxidant capacity of blood of rats. The antioxidant capacity of blood of healthy rats was higher than that of rats which received only tramadol and were exposed to electromagnetic fields.

Key words:
G protein-coupled receptors, tramadol, electromagnetic field, blood antioxidant capacity, fluorescence-based oxygen radical absorbance capacity (ORAC-FL)

Abbreviations: AAPH – 2,2-azobis(2-amidinopropane) dihydrochloride, ANOVA – analysis of variance, AUC – area under the curve, CFA – Complete Freund's Adjuvant, EMF – electromagnetic field, GABA – γ-aminobutyric acid, GPCR – G protein-coupled receptor, GSH-Px – glutathione peroxidase, GSM – Global System for Mobile Communications, MPO – myeloperoxidase, MRI – magnetic resonance imaging, MX – 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, NMDA – N-methyl-D-aspartate, ORAC – oxygen radical absorbance capacity, ORAC-FL – fluorescence-based ORAC assay, PMA – phorbol-12-myristate-13-acetate, RF – radio frequency, ROS – reactive oxygen species, SAR – specific absorption rate, SNO – S-nitrosothiol, SOD – superoxide dismutase, TRAM – tramadol

Introduction

Oxidative stress is defined as an imbalance between production of reactive oxygen species (ROS) and a biological system's ability to neutralize them *via* antioxidant enzymatic and/or non-enzymatic activity. Large amounts of reactive intermediates lead to cell component damage and production of secondary toxic compounds e.g., reactive aldehydes and ketones [1, 37] and, as a consequence, causing increased risk of many diseases, including cancer.

A number of systems are involved in eliminating free radicals and other ROS from the body, but such mechanisms are not entirely efficient [39, 41].

The respiratory process is the source of ROS production, with subsequent ROS reactions with intracellular DNA and lipoprotein leading to altered cellular function. ROS include very small and highly reactive species such as: oxygen ions, free radicals, and both inorganic and organic peroxides. The harmful cellular effects, such as damage of DNA, oxidation of polyunsaturated fatty acids in lipids, and oxidation of amino acids in proteins, are closely related with ROS. It was stated that ROS, such as peroxyl radicals ($ROO^{\bullet}$), hydroxyl radicals ($HO^{\bullet}$), superoxide ion ($O_2^{-\bullet}$), and singlet oxygen ($^1O_2$), are involved in the pathophysiology of aging and several diseases, such as cancer, Alzheimer's disease, and Parkinson's disease [7, 9].

Selected studies have presented a link between human health and exposure to electromagnetic field (EMF), with an emphasis on various clinical conditions, including childhood leukemia, genotoxicity, brain tumors, and neurodegenerative diseases [14, 21].

In an attempt to explain these effects, which are not yet fully understood, it has been suggested that oxidative stress could be a key factor [11]. However, a review [16] of the significance of oxidative stress in mediating the pathological effects of EMF in cell lines has provided contradictory results, possibly due to differences in the underlying redox susceptibility of varied cell lines.

We would like to emphasize that there are only a few studies, directed particularly toward male fertility, which have examined the effects of EMF fields on ROS production [24, 32, 40].

Lantow et al. examined the effect of EMF (GSM 1800 MHz, specific absorption rate (SAR) = 0.5–2.0 W/kg) on ROS production. Heat and PMA (phorbol-12-myristate-13-acetate) treatment induced a significant increase in superoxide radical anions and in ROS production in the applied tissue culture (Mono Mac 6) [24].

There were no significant differences in the production of free radicals detected after exposure to EMF or in appropriate controls, and no additional effect on superoxide anion radical production was detected after co-exposure to EMF + 12-O-tetradecanoylphorbol-13-acetate (TPA) or EMF + lipopolysaccharide (LPS).

Zeni et al. [40] studied the induction of ROS in murine L929 fibrosarcoma cells, which were exposed to EMF at 900 MHz, with or without co-exposure to 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), a potent environmental carcinogen produced during chlorination of drinking water. This study provided no indication that 900-MHz EMF exposure, either alone or in combination with MX, induced formation of ROS under any of the investigated experimental conditions.

At present, no study has reported that ROS production is directly increased by EMF exposure.

Therefore, observing the possible influence of high-frequency electromagnetic fields (HF-EMFs) on a defense mechanism against oxidative stress which may occur as a result of a drug administration and application of electromagnetic field in conditions of health and disease, was a primary aim of our study. The present research is a continuation of our studies [3] which showed that a HF-EMF of 1800 MHz did not influence pain threshold to thermal stimulus in either normal or inflammatory state conditions, but attenuated analgesic action of tramadol. We expanded the scope of our previous findings by evaluating the antioxidant capacity of blood samples of the examined rats.

Taking into account the various defense mechanisms against free radicals that may exist in the blood, we used the most wide-ranging assessment, namely

oxygen radical absorbance capacity (ORAC), well known and widely used in the examination of antioxidative capacity for a broad spectrum of foods and biological samples e.g., body fluids. The ORAC assay determines the degree of inhibition of peroxy-radical-induced oxidation of a fluorescent probe by the compounds of interest in a chemical milieu. It includes both inhibition time and the extent of inhibition of oxidation [32].

The ORAC assay is considered to be a preferable method because of its biological relevance to the *in vivo* antioxidant efficacy [2].

Decay curves (fluorescence intensity *vs.* time) are recorded and the difference of the area under the two decay curves (with or without antioxidant) is calculated. Subsequently, the degree of antioxidant-mediated protection is quantified using the antioxidant Trolox (a water-soluble vitamin E analogue) as a standard. Results for test samples have been published as Trolox equivalents (TE) [12, 17].

Material and Methods

Animals

Experiments were performed on male Wistar rats weighing 220–250 g purchased from Center of Experimental Medicine (Medical University of Bialystok, Poland). Animals were housed in cages on a standard 12 : 12 h light/dark cycle. Water and food were available *ad libitum* until rats were transported to the laboratory, approximately 1 h before experiments. Animals presenting any symptoms of illness were excluded from the study. All testing was performed between 9:00 a.m. and 4:00 p.m. and the animals were used only once. The experimental protocol was approved by the IV Local Ethics Committee for Animal Experimentation.

Drugs and chemicals

Complete Freund's Adjuvant (CFA; heat killed *Mycobacterium tuberculosis* suspended in paraffin oil, 1 mg/ml) and paraffin oil were purchased from Sigma-Aldrich.

Tramadol hydrochloride (Tramal®, Grünenthal, Germany) was used in the form of injectable solution in aqua for injection, 20 mg/kg body mass, by intraperitoneal route.

2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich, and uranin (fluorescein sodium salt), potassium chloride, sodium chloride, monopotassium phosphate and dipotassium phosphate were from POCh (Gliwice, Poland).

Experimental procedure

Experiments were performed on healthy rats, and on rats with persistent inflammatory state, induced by CFA injection.

Persistent inflammation was elicited 24 h before EMF exposure and drug application. Rats (n = 10) were injected with 0.1 ml volume of CFA or paraffin oil into the plantar surface of the left hind paw.

Animals were placed in pairs in Plexiglas enclosures positioned centrally, 1 meter from the EMF source, and exposed to the far-field range of an antenna at 1800 MHz with the additional modulation identical to that generated by mobile phone GSM 1800, and the value of effective electric field 20 V/m and effective magnetic field value 0.05 A/m. The propagation vector of the incident wave was parallel to the long axis of the animal's body. Rats were given 15 min exposure or were sham-exposed with no voltage applied to the field generator in control groups. Immediately before EMF exposure, particular groups of rats (n = 10) were injected intraperitoneally with TRAM in the 20 mg/kg dose or vehicle (*aqua pro injectione*) in the 1 ml/kg volume.

After the experiments, the rats were subjected to general anesthesia induced by intraperitoneal injection of ketamine (Bioketan 10 mg/kg, Vetoquinol Biowet) and xylazine (Xylapan 10 mg/kg, Vetoquinol Biowet) and blood samples were collected by incision of the jugular vein. The rats were then euthanized by pentobarbital sodium overdose (Morbital, 200 mg/kg, Biowet, Poland). Serum samples were frozen at –70°C and left to further examination of antioxidant capacity estimated by ORAC method.

ORAC assay

Oxidative stress can be monitored in body fluids by various analytical methods, including estimation of total antioxidant capacity by ORAC. The ORAC-

fluorescein (ORAC-FL) assay was based on the procedure of Ou et al. [29].

The ORAC-FL test uses fluorescein as a fluorophore [8] and follows the decrease of the fluorescence intensity resulting from exposition to peroxyl radical generated with AAPH as a radical source. This decay is substantially delayed in the presence of antioxidants [7, 22].

All solutions were prepared daily in PBS buffer, pH 7.4. For measurements, 30 µl of 300-fold diluted serum, standard solution or, in case of a blank, PBS buffer, and 180 µl of 112 nM fluorescein solution were mixed in a well of 96-well microplate and thermostated for 10 minutes at 37°C. Then, 100 µl of 100 mM AAPH solution was added and fluorescence was measured every minute for 90 min with the fluorescence spectrometer Hitachi F-7000 equipped with microplate accessory, at 37°C. For each plate the standard curve was prepared with Trolox concentrations in the range of 10–100 µM. All measurements were made in triplicate.

ORAC value, expressed in Trolox equivalents (TE) (µmol Trolox/100 ml), was determined with the standard curve – net AUC *vs.* Trolox concentration, where net AUC is the difference between area under the fluorescence decay curve for the sample or standard solution and a blank. AUC was calculated according to the equation:

$$AUC = 1 + f_1/f_0 + f_2/f_0 + ... + f_{90}/f_0,$$

where f_i is fluorescence intensity in i minute and f_0 is the initial fluorescence intensity.

Results

Our study revealed that single EMF exposure in 1800 MHz frequency significantly reduced antioxidant capacity both in healthy animals ($p < 0.00001$, Fig. 1) and those with paw inflammation ($p < 0.01$, Fig. 2). Similar effect on ORAC value was observed after TRAM injection in normal and inflammatory state conditions. In healthy rats treated with TRAM, EMF exposure slightly increased ORAC value (Fig. 1). The same, but statistically significant effect was observed in rats with paw inflammation and TRAM injection ($p < 0.01$, Fig. 2).

For statistical evaluation, the one-way analysis of variance ANOVA for comparison within each group of rats (healthy and CFA-treated) and the two-way ANOVA for comparison of both groups were applied. Significance between the groups was verified with NIR, Bonferroni and Scheffé methods (StatSoft, Inc., 2008). STATISTICA (data analysis software system), version 8.0. www.statsoft.com, and GraphPad Prism software, version 5.01 (www.graphpad.com).

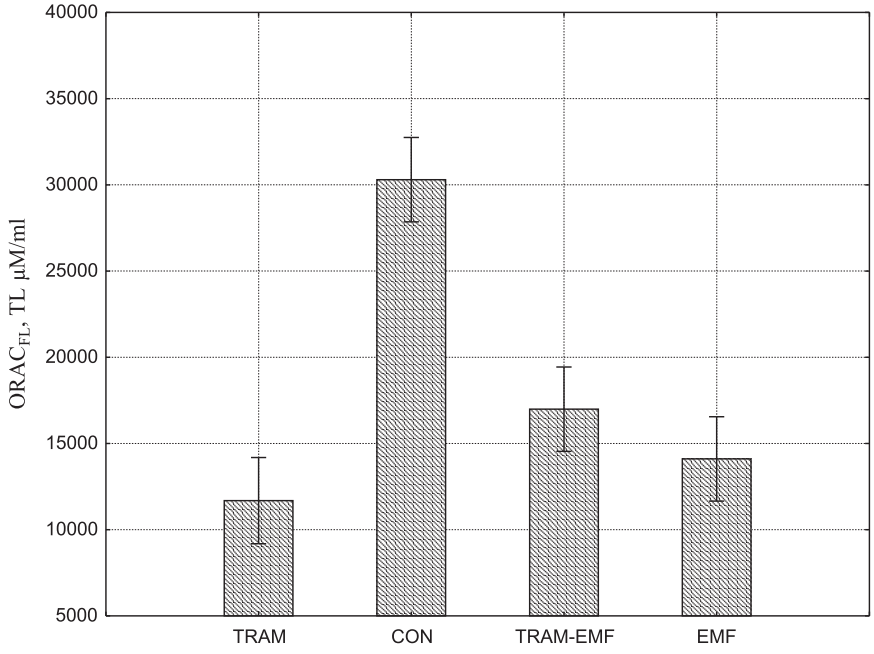

Fig. 1. Oxygen radical absorbance capacity ($ORAC_{FL}$) of the blood of healthy rats treated with synthetic opioid drug tramadol and exposed to electromagnetic field (1800 MHz). TRAM – group treated with the drug and not exposed to electromagnetic field, EMF – group without the drug and exposed to electromagnetic field, CON – control group without the investigated factors, TRAM-EMF – group treated with the drug and exposed to electromagnetic field. Results are shown as the mean ± SEM; $p < 0.0001$ TRAM-EMF *vs.* CON and $p < 0.00001$ TRAM *vs.* CON

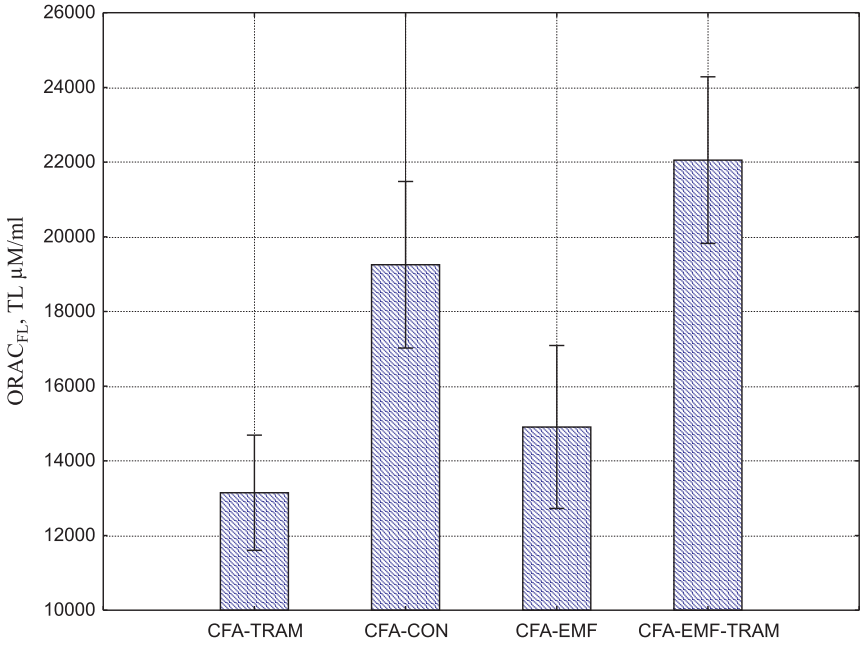

Fig. 2. Oxygen radical absorbance capacity (ORAC$_{FL}$) of the blood of rats treated with an inflammatory factor (CFA) and synthetic opioid drug tramadol and exposed to electromagnetic field (1800 MHz). CFA-TRAM – group which received the drug and not exposed to electromagnetic field, CFA-CON – group which received inflammatory factor (CFA) only. CFA-EMF – group which did not receive the drug, but exposed to the electromagnetic field. CFA-EMF-TRAM – group with the drug and exposed to electromagnetic field. Results are shown as the mean ± SEM; $p < 0.05$ CFA-EMF *vs.* CFA-EMF- TRAM

Statistically significant differences were seen between control groups ($p < 0.05$), and between the groups subjected to TRAM injection and EMF exposure.

Discussion

Our environment is permeated by electromagnetic fields (EMFs) originating from both natural and man made sources.

A matter of great debate is whether people who are continuously exposed to EMFs in everyday life may be susceptible to harmful effects.

There is another important question – namely, whether the mutual effect can occur during simultaneous influence of EMFs and the central nervous system drugs, such as opioids.

Oxidative stress may be generated by EMFs' interaction with biological systems under certain circumstances.

Tramadol is a centrally acting opioid analgesic with additional ion channel blocking activity, supposedly possessing NMDA antagonistic and GABA agonistic properties.

A recently presented study revealed the possible neuroprotective effect of tramadol hydrochloride in a rat model of transient forebrain ischemia [27]. Tramadol pretreatment attenuated the post ischemic motor impairment and significantly reduced ($p < 0.001$) the extent of lipid peroxidation, which was higher in the ischemic control group.

Free radicals are essential for brain physiological processes and pathological degeneration.

The EMFs-driven oxidative stress research is still in progress, especially regarding the recent studies, suggesting that EMFs may contribute to neurodegenerative disorders.

EMFs are widely produced, not only for technological applications such as power lines and mobile phones, but also widely used in medicine for diagnostic purposes e.g., magnetic resonance imaging (MRI) and therapeutic purposes in cosmetic medicine e.g., radiofrequency and microwave ablation and hyperthermia.

EMFs' influence may be explained by two distinct interaction mechanisms: thermal effects, based on the ability of radio frequency (RF) fields to transfer their energy to biological matter, increasing the tissue temperature through the vibration of molecules, and non-thermal effects [10, 13].

Only non-thermal effects have been correlated to the generation of oxidative stress.

Non-thermal effects may cause alterations in the permeability of the blood-brain barrier, and changes in encephalogram and blood pressure [33], and require further thorough studies.

There is still lack of a theoretical basis of non-thermal effects of EMFs and how to distinguish them from direct and indirect thermal effects.

Oxidative stress has been proposed supposedly as the underlying mechanism responsible for this kind of RF effects.

It has been proposed that EMF (875 MHz, 0.07 mW/cm^2) may generate extracellular ROS by stimulating cell membrane nicotinamide adenine dinucleotide (NADH) oxidase in rats and HeLa cells *in vitro* [11].

RF exposure induces lipid peroxidation, accompanied by decreased activity of superoxide dismutase (SOD), myeloperoxidase (MPO) and glutathione peroxidase (GSH-Px), which has been reported in various organs, such as guinea pigs liver and rat kidney [30].

However, no significant ROS generation was measured in human cell lines when exposed to 1800 MHz (0.5–2 W/kg, for 30–45 min) [23].

In other studies, conducted on rats' models, authors obtained contrary results on the effects of EMFs on analgesia induced by morphine and other opioid compounds.

Research on the simultaneous influence of analgesic opioid drugs and environmental factors on free radicals' scavenging mechanisms during pain treatment may prove to be significant.

Inflammation, which is a result of tissue destruction, abnormal immune reactivity or nerve injury, is often associated with acute and chronic pain. Neuronal-glial communication and production of pro-inflammatory cytokines during hyperalgesic states is highly dependent on leakage of reactive oxygen species from mitochondria, which are highly relevant signaling events [31].

Peripheral terminals of afferent fibers contain opioid receptors and the axonal transport of opioid receptors is increased during inflammation. ROS such as nitric oxide may appear in larger quantities during hyperalgesic activation states in oxidative stress conditions [36].

We suppose that in accretion of antioxidative abilities of blood of rats, exposed to electromagnetic field and simultaneous administration CFA and tramadol, G-proteins may be involved in a potential mechanism.

A family of seven transmembrane receptors, known as G protein-coupled receptors, are engaged in several transduction pathways, which are based on heterotrimeric G proteins (G proteins), which play an important role as their integral component.

G protein-coupled receptors (GPCRs) are available in numerous tissues, and their dysfunction may influence a broad range of diseases.

The biological responses mediated by GPCRs are not purely initiated at the cell surface, but may result from the integration of extracellular and intracellular signaling pathways. GPCRs may be activated by a variety of factors, including electromagnetic field, neuropeptides, nucleosides/nucleotides, hormones, calcium ions, chemokines, biogenic amines, proteases, lipids or fatty acid mediators. GPCRs change these signals into intracellular molecular responses [15].

Nuclear-localized receptors are involved in regulation of the distinct signaling pathways. Extensive evidence shows that various G protein-coupled receptors form signaling complexes with voltage-gated calcium channels and regulate their membrane expression [19, 20, 25, 34].

The opioid and opioid-like receptors play a key role in controlling pain signaling, in primary afferent fibers, by two primary mechanisms [4, 5, 28]. Firstly, G protein-coupled inward rectifier potassium channels are activated by opioid receptors and neuronal excitability [6, 26] and are reduced, secondly N-type calcium channels in nerve terminals within the dorsal horn of the spinal cord are inhibited [38].

G protein-coupled inwardly rectifying K^+ channels (GIRK), affected by the EMF, can play a major role in inhibitory signalling in excitable and endocrine tissues [18].

The gating mechanism of K^+ channels is mediated by a direct interaction of the subunits of G protein – Gbγ, which are released upon inhibitory neurotransmitter receptor activation. This mechanism is manifested in the next stages by intracellular factors such as anionic phospholipids and Na^+ and Mg^{2+} ions, which may also be susceptible to exposure of applied electromagnetic fields.

GPCRs activation in vascular cells involves generation of ROS, including superoxide (O_2^-) and H_2O_2 as well as reactive nitrogen species (RNS), including nitric oxide and its biological metabolites *S*-nitrosothiols (SNOs) [35].

Conclusion

We recorded interesting findings for the groups of rats receiving tramadol and simultaneously exposed to an electromagnetic field.

The ORAC values in the group treated with tramadol were significantly lower than in other groups (Fig. 1). However, in the groups treated with inflam-

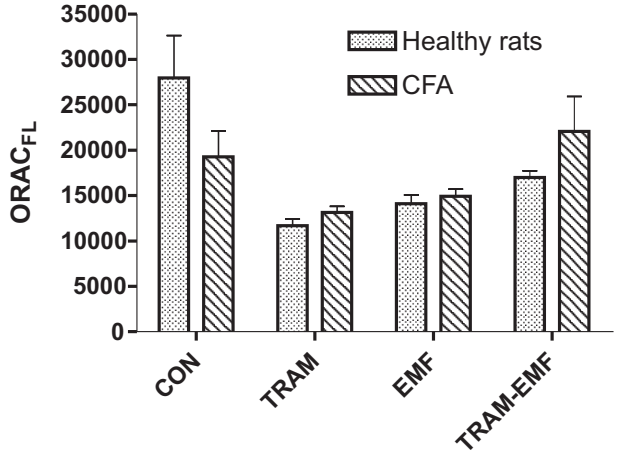

Fig. 3. Comparison of mean ORAC$_{FL}$ values in sera of healthy rats and rats with persistent inflammatory state (CFA). Results are shown as the mean ± SEM; $p < 0.05$ TRAM-EMF *vs.* CON

matory factor – antioxidant capacity in CFA/EMF/TRAM group did not differ from CFA/control.

We observed a certain synergic mode of action between applied EMFs and administered tramadol in rats treated with CFA. The antioxidant capacity of blood of healthy rats was higher than that of rats which received only tramadol, and were exposed to EMFs.

An explanation of the mechanism of mutual influence of EMF and tramadol on blood antioxidant capacity, especially in the presence of inflammatory conditions, requires further studies.

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